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Improving the Accuracy of ^{1}H-^{19}F Internuclear Distance Measurement using 2D ^{1}H-^{19}F HOESY

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Abstract

With the rise in fluorinated pharmaceuticals it is becoming increasingly important to develop new ^{19}F NMR-based methods to assist in their analysis. Crucially, obtaining information regarding the conformational dynamics of a molecule in solution can aid the design of strongly binding therapeutics. Herein we report the development of a 2D ^{1}H-^{19}F HOESY experiment to measure ^{1}H-^{19}F internuclear distances, with accuracies of ~5% when compared to ^{1}H-^{19}F internuclear distances calculated by quantum chemical methods. We demonstrate that correcting for cross relaxation of ^{1}H, using the diagonal peaks from the 2D ^{1}H-^{1}H NOESY, is critical in obtaining accurate values for ^{1}H-^{19}F internuclear distances. Finally, we show that by using the proposed method to measure ^{1}H-^{19}F internuclear distances we are able to determine the relative stereochemistry of two fluorinated pharmaceuticals.

Key Words: NMR, ^{1}H, ^{19}F, HOESY, PANIC, Quantitative ^{1}H-^{19}F Distances

Introduction

The development of new ^{19}F NMR-based methods is being driven by the growth in newly approved small molecule drugs containing at least one fluorine atom[1] arising from the beneficial pharmaceutical effects when a hydrogen is replaced with a fluorine including improvements to conformation, potency and metabolic stability.[2] ^{19}F NMR is also highly attractive as a tool for these systems with 100% natural abundance, I=½, high gyromagnetic ratio and minimal spectral overlap (spectral width of ^{19}F is >400 ppm, with typically only a handful of ^{19}F environments in a given molecule).[3]

Recently there has been growing emphasis on understanding the conformational bias of both the unbound and bound state of a drug molecule in the design of strongly binding therapeutics.[4] The combination of chemical shift, scalar coupling and internuclear distance information from NMR spectroscopy is key to this understanding and maximizing the accuracy of such measurements increases the precision of any such study. Of particular relevance here is the use of through-space interproton contacts from ^{1}H-^{1}H NOESY experiments, helping to elucidate molecular structure, conformation and relative stereochemistry.[5–10] Arguably this technique is the most valuable experimental technique for measuring the crucial conformational dynamics of drug molecules in solution, due to the strong non-linear distance dependence which can make it sensitive to vanishingly low levels of minor conformers.[11]

We have recently demonstrated that ^{1}H-^{1}H NOESY analysis of small molecules can give accurate quantitative interproton distances, building on improvements in NMR hardware, NOE experimental methods and data analysis.[11,12] Specifically, the measurement of interproton distances involves a comparison between the relative NOE intensities for spin pairs in transient NOESY experiments. Provided that the molecule is in the fast tumbling regime ($\omega\tau_C << 1$) and that measurements are made within the initial rate approximation limits, then the ratio of a pair of NOE intensities is proportional to the ratio of their internuclear distances (*vide infra*).[13][14] The key weakness of this approach is the assumption of the initial rate approximation *i.e.* that the NOE build-ups of the spins are not affected by external relaxation. There are numerous approaches to addressing this assumption, but probably the simplest is the use of PANIC (Peak Amplitude Normalization for Improved Cross Relaxation).[15,16] PANIC corrects the experimental NOE intensities by standardization against the irradiated or diagonal peak in the 1-dimensional or 2-dimensional NOESY experiments respectively – effectively assuming that the external relaxation rates that perturb the NOE signal are dominated by the external relaxation of the irradiated/diagonal peak. Using PANIC, ^{1}H-^{1}H distances can be measured with accuracies of +/-3% in ideal systems. With an increasing number of fluorinated pharmaceuticals being developed, measuring the ^{1}H-^{1}H distances of a molecule is not always sufficient to understand the conformation of a drug molecule. Measurement of ^{1}H-^{19}F distances instead offers a complimentary and useful approach. In 2012, Claridge *et. al.* reported the measurement of ^{1}H-^{19}F distances using a 1D ^{19}F-^{1}H HOESY (Heteronuclear Overhauser Spectroscopy) experiment,[17] but did not apply PANIC – presumably for the simple reason that the irradiated ^{19}F signal does not

appear in the measured ^{1}H spectrum. Consequently, heteronuclear NOE build-up curves were required to identify the region in which the initial rate approximation holds true.

This work demonstrates how PANIC can be applied to the measurement of ^{1}H-^{19}F internuclear distances, avoiding the need for NOE build-up curves. Instead, we propose using the diagonal peaks of the 2D ^{1}H-^{1}H NOESY to correct the intensities in a 2D ^{1}H-^{19}F HOESY experiment. Although this requires the acquisition of both a 2D ^{1}H-^{19}F HOESY and a 2D ^{1}H-^{1}H NOESY, all possible ^{1}H-^{19}F internuclear distances that can be observed by NOE will be extracted.

Results and Discussion

Voriconazole, a commercially available antifungal medicine featuring three aromatic fluorine nuclei, was used to develop the proposed method.[18] We measured a ^{1}H-observed 2D ^{19}F-^{1}H HOESY (Figure 1b), putting ^{19}F in the low digital resolution 'indirect' dimension, which revealed a total of 12 measurable ^{1}H-^{19}F NOE correlations within the molecule. The pulse sequence used was that reported by Bauer in 1996.[19] Full experimental details are provided in the supporting information. A 2D ^{1}H-^{1}H NOESY (Figure 1a) was also acquired with equal mixing time, to provide the diagonal peaks needed to correct for relaxation differences of the observed nucleus (both spectra are shown in Supporting Information).

By analogy with our previous ^{1}H-^{1}H internuclear distance measurements, assuming that the initial rate approximation is true, and that the molecule is tumbling isotropically in solution, the internuclear ^{1}H-^{19}F distances should be measurable by comparing the PANIC-corrected HOESY intensities (η), via Equation (1)

$$r_{HF(target)} = r_{HF(reference)} \left[\frac{\eta_{HF}(target)}{\eta_{HF(reference)}}\right]^{-1/6} \quad (1)$$

Where $r_{HF(target)}$ is the desired internuclear distance between the target ^{1}H-^{19}F pair, $r_{HF(ref)}$ is the distance between a 'reference' ^{1}H-^{19}F pair with a known internuclear separation (best chosen for a pair where the internuclear distance is not affected by conformational dynamics), and $\eta_{HF(target)}$ and $\eta_{HF(ref)}$ are the corresponding PANIC-corrected HOESY intensities. The PANIC-corrected intensities are obtained from the experimental spectra by multiplying the target ^{1}H-^{19}F NOE intensity by a scaling factor, *f*, obtained from the ratio of the corresponding target protons ^{1}H-^{1}H diagonal peak intensity against a chosen (arbitrary) standard diagonal peak intensity.

For example, to PANIC-correct the H12''-F9 NOE intensity (Figure 1b, η = 0.06), we first calculate the PANIC scaling factor for H12'' by comparing the absolute intensity of the ^{1}H-^{1}H-NOESY diagonal peak for H12'' (Figure 1a, D=2.93) to that of (arbitrarily chosen) H14 (Figure 1a, D=4.74). The ratio of these intensities, 2.93/4.74, gives the corresponding PANIC scaling factor for H12, $f_{H12''}$, of 0.6174. This means that H12'' relaxes faster than H14 and thus the intensity of any H12'' HOESY peaks should be scaled up to correct for this relaxation. Scaling up the absolute intensity of the H12''-F9 HOESY cross peak (0.06) by $1/f_{H12''}$ gives the PANIC-corrected intensity, $\eta_{H12''\text{-}F9}$, of 0.10 (see highlighted row of Table 1).

To obtain the H12''-F9 internuclear distance, we simply now apply Equation 1, using a reference NOE intensity and distance from another ^{1}H-^{19}F pair. As the H21-F23 NOE arises from a fixed internuclear distance (2.55 Å), which is independent of the molecular conformation, it can be used as a sensible reference pair. *NOTE:* To provide a best fit for each dataset, the experimentally determined reference NOE-distance H21-F23 is subsequently incremented to maximize the quality of fit between experimental and computed distances (see SI for full details). The PANIC-correction for the experimental intensity of the reference H21-F23 NOE (1.26) is obtained from the absolute intensity of the H23 ^{1}H-^{1}H NOESY diagonal peak (4.857) compared to that of H14, which gives f_{H21} = 1.024, and thus a PANIC-corrected intensity, $\eta_{H21\text{-}F23}$ of 1.23. Plotting the NOE build-up curves for these PANIC-corrected intensities vs their corresponding uncorrected intensities shows substantial improvement in linearity (illustrative plots are shown in section 5.4 is Supporting Information), highlighting a key source of NOE-distance errors in uncorrected data. Now applying the values to Equation 1 (*i.e.* $r_{HF(ref)}$ = 2.55 Å, $\eta_{HF(ref)}$ = 1.23, $\eta_{H12''\text{-}F9}$ = 0.10) we thus calculate the H12''-F9 distance to be 3.89 Å (see highlighted row in Table 1). Extending this approach to all other ^{1}H-^{19}F NOE pairs in the 2D ^{1}H-^{19}F HOESY spectrum (using the H14 ^{1}H-^{1}H diagonal intensity and H21-F23 reference NOE intensity in all cases), a full set of ^{1}H-^{19}F internuclear distances are obtained for Voriconazole.

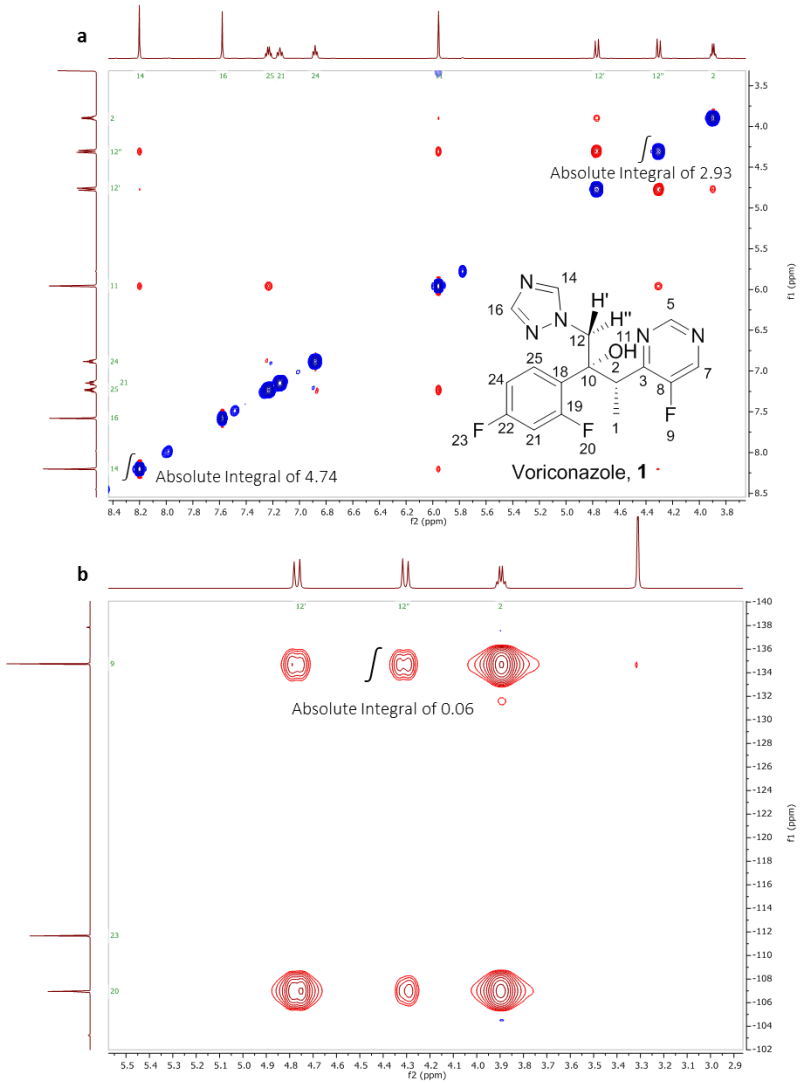


Figure 1. **a)** The ^{1}H-^{1}H NOESY of Voriconazole showing the absolute integral of the reference peak H14 and the peak of interest H12'' **b)** The ^{1}H-^{19}F HOESY of Voriconazole showing the absolute integral of the peak of interest H12''-F9.

In order to determine the accuracy of the PANIC-corrected internuclear distance measurements, we compared the experimentally derived NOE-distances to those predicted for Voriconazole by DFT calculations. An initial conformational search of Voriconazole, using molecular mechanics, found a total of 21 non-redundant conformations under 21 kJmol^{-1}. All 21 conformations were geometry optimized at the mpw1pw91/6-31g(d) level of theory and frequency calculations were

performed for each. A subsequent geometry optimization and single-point energy calculations were performed at higher level (mpw1pw91/6-311g(d,p)). The lower level frequency corrections were then applied to the higher level single-point energies to establish the free energy for each conformer. The contribution to each NOE arising from each conformer (derived the negative 6th power of the corresponding ^{1}H-^{19}F distance, $\eta_{HF} \alpha r_{HF}^{-6}$, in each conformer) were then population-averaged using estimated Boltzmann populations from the free energies of the conformer ensemble, before conversion back to a single effective internuclear NOE-distance averaged across all conformers ($r_{HF} \alpha \eta_{HF}^{-(1/6)}$). Full computational details are provided in the supporting information.

The calculated NOE-distances, r_{HF}, were then compared to the experimentally derived values and it was found that using the PANIC-corrected HOESY intensities (column 6, Table 1) leads to a good fit between experimental and DFT-determined NOE-distances (5.9% MAD, 7.2% StDev) with a maximum deviation of 16.1% for the distance between H2 and F20. These deviations, while not as good as the ~3% values obtained for ^{1}H-^{1}H distances in rigid molecules, are in line with those we have observed previously for conformationally flexible systems with errors arising from DFT energy/population estimations and non-r^{-6} NOE kinetics of dynamic systems.[5,7] Crucially, ignoring PANIC and using the uncorrected HOESY intensities (column 4, Table 1) gave a substantially less good fit (MAD 8.0%, StDev 9.7%) with maximum deviation of 22.5% for H12' to F20.

Table 1. The determination of the ^{1}H-^{19}F distances in Voriconazole using PANIC corrected 2D ^{1}H-^{19}F HOESY

2D ^{1}H-^{1}H NOESY		2D ^{1}H-^{19}F HOESY					
H	Diagonal Peak Intensity	F	Uncorrected		PANIC-corrected		DFT calc. r_{HF}
			η_{HF}	r_{HF} / Å	η_{HF}	r_{HF} / Å	
1	9.08	9	0.11	3.84	0.06	4.26	3.99
2	4.39	9	0.88	2.71	0.95	2.66	2.43
7	4.34	9	0.98	2.66	1.08	2.61	2.61
12'	2.83	9	0.07	4.09	0.12	3.74	3.89
12''	2.93	9	0.06	4.23	0.10	3.89	4.02
1	9.08	20	0.10	3.88	0.05	4.31	4.20
2	4.39	20	0.70	2.81	0.76	2.76	2.32
12'	2.83	20	0.45	3.03	0.75	2.77	2.35
12''	2.93	20	0.04	4.57	0.06	4.20	3.81
21	4.86	20	1.35	2.52	1.32	2.52	2.56
21	4.86	23*	1.26	2.55	1.23	2.55	2.60
24	4.93	23	1.27	2.55	1.22	2.56	2.59
14*	4.74						
* Denotes a reference peak or distance							
Mean Absolute Deviation, MAD (%)			**8.02**		**5.91**		
Standard Deviation, StDev (%)			**9.67**		**7.15**		

The PANIC correction applied here (^{1}H-PANIC to the ^{1}H-observed 2D ^{1}H-^{19}F HOESY) is only one of a number of possible combinations that could be applied to ^{1}H-^{19}F interproton distance measurements using NOE. Consequently, we compared the four combinations against each other:

- ^{1}H-PANIC correction (from the ^{1}H-^{1}H NOESY diagonal) to the ^{1}H-observed HOESY (described above)
- ^{19}F-PANIC correction (from the ^{19}F-^{19}F NOESY diagonal) to the ^{1}H-observed HOESY
- ^{1}H-PANIC correction (from the ^{1}H-^{1}H NOESY diagonal) to the ^{19}F-observed HOESY
- ^{19}F-PANIC correction (from the ^{19}F-^{19}F NOESY diagonal) to the ^{19}F-observed HOESY

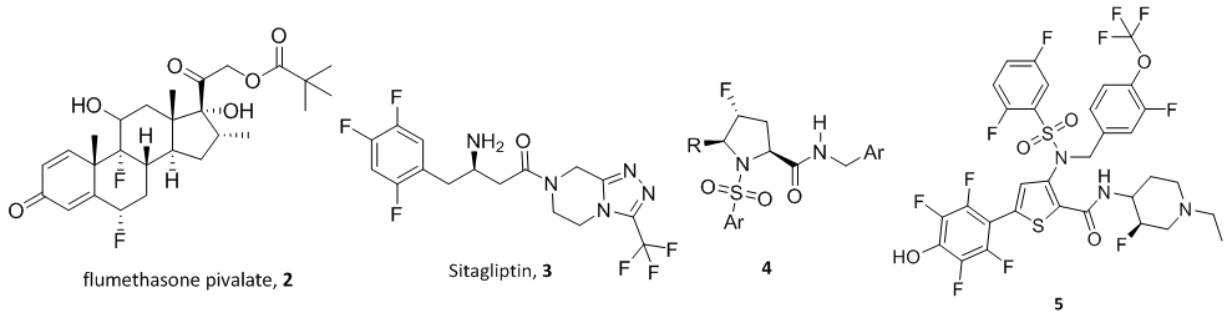
The ^{19}F-PANIC correction based on the diagonal peak of the 2D ^{19}F-^{19}F NOESY to correct for cross-relaxation of the ^{1}H-observed 2D ^{1}H-^{19}F HOESY resulted in a negligible improvement to the deviations between experimental and calculated internuclear distances (Table 2, column 3). This suggests that correcting for variations between cross relaxation of the ^{1}H spins is more helpful than correcting for these between the ^{19}F spins. Supporting this, T_1 measurements (see Supporting Information) show a much greater variability in T_1 for ^{1}H (from 0.38s for H11 to 7.39s for H5) than for ^{19}F (from 0.32s for F20 to 0.78s for F23).

The same general trend (^{1}H-PANIC is more helpful than ^{19}F-PANIC) was observed for NOE-distances extracted from the ^{19}F-observed HOESY (Table 2, columns 4 and 5), however in this case the improvement over the uncorrected NOE-distances was relatively negligible in either case, with a best MAD of 7.36% obtained for ^{1}H-PANIC corrected distances compared to DFT.

Table 2. A comparison of the MADs obtained when applying the possible PANIC corrections to the ^{1}H-^{19}F internuclear distances.

^{1}H-Observed ^{1}H-^{19}F HOESY			**^{19}F-Observed ^{1}H-^{19}F HOESY**		
MAD before PANIC	MAD after PANIC using the ^{1}H-^{1}H NOESY (%)	MAD after PANIC using the ^{19}F-^{19}F NOESY (%)	MAD before PANIC	MAD after PANIC using the ^{1}H-^{1}H NOESY (%)	MAD after PANIC using the ^{19}F-^{19}F NOESY (%)
8.02	5.91	7.72	8.52	7.36	8.28

We then applied ^{1}H-PANIC for ^{1}H-^{19}F HOESY NOE-distance measurements to a number of other fluorinated drug-like molecules **2-5** of varying flexibility and complexity (Figure 2). There were significant improvements in the MAD/StDev upon ^{1}H-PANIC correction **3**, **4**, and **5**, although only marginal changes were observed for the Flumethasone pivalate **2** where the fit was already very good without PANIC.



	Before PANIC Correction		After PANIC Correction	
	MAD (%)	StDev (%)	MAD(%)	StDev (%)
Flumethasone Pivalate, **2**	4.17	5.13	3.87	5.69
Sitagliptin, **3**	11.57	11.30	5.49	7.77
4	7.78	8.03	3.78	5.27
5	5.91	8.85	4.02	5.92

Figure 2. A comparison of the MAD and StDev for drug like molecules **2-5** before and after the proposed PANIC correction.

A key application of internuclear distance measurements are the determination of relative stereochemistry and/or conformation. To examine this, we calculated the ^{1}H-^{19}F NOE-distances for alternative diastereomers of both voriconazole and **4**. In both cases (Figure 3) the calculated NOE-distances for the alternative diastereomers give substantially worse fits to the PANIC-corrected experimental NOE-distances (voriconazole_*RR:* MAD 10.7%, StDEV 15.6%; **4**_*S*: MAD 14.1%, StDEV 18.3%). Importantly the discrimination between the correct and incorrect diastereomers for both voriconazole and **4** is <<1-fold without PANIC correction, and improves to ≥2-fold when using the PANIC-corrected distances. While in both cases the discrimination might still be made without PANIC correction, the level of confidence in this discrimination is substantially lower. This better quality of fit for the correct diastereomers validates the value of maximizing the accuracy and sensitivity to molecular structure with PANIC-corrected analysis.

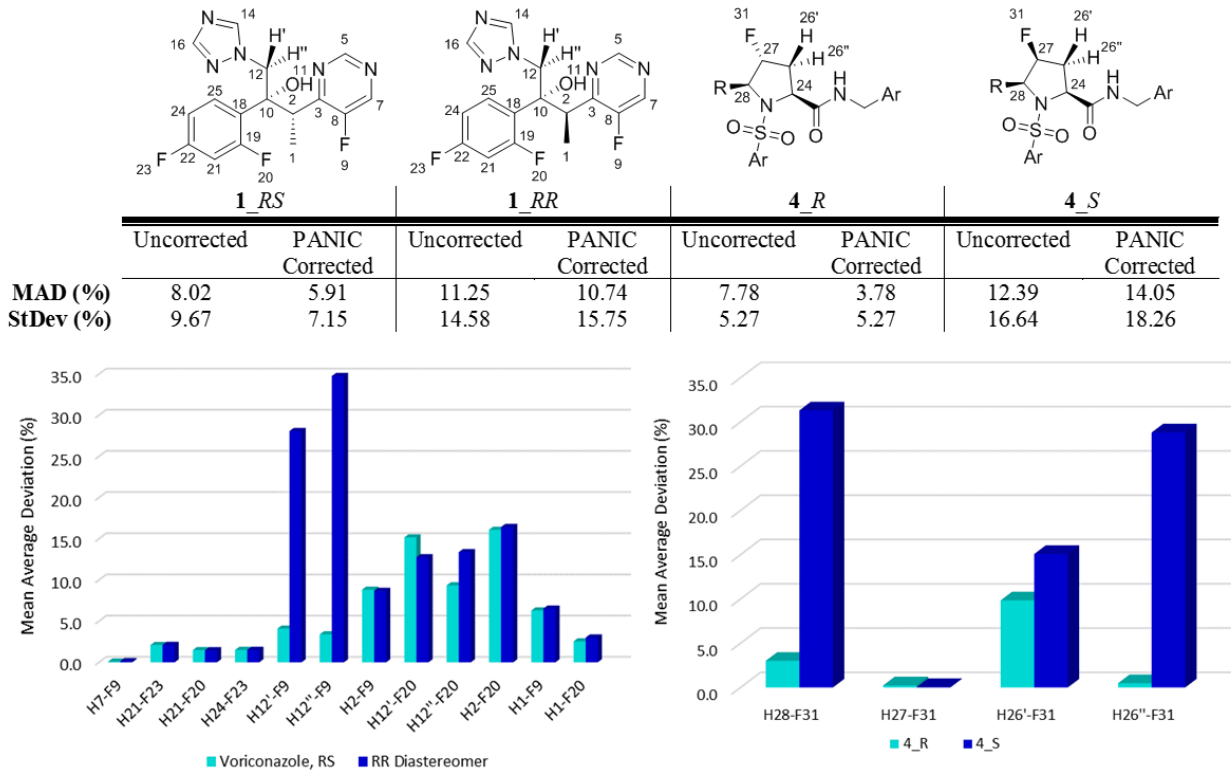


Figure 3. A comparison of the MAD and StDev obtained when comparing the experimental HF distances obtained to those calculated for two diastereomers of **1** and **4**. The graphs below show the MAD of discriminating HF distances that allow relative stereochemistry to be confidently assigned.

Conclusions

In summary, the accuracy of ^{1}H-^{19}F NOE-distance measurements can be maximized by the ^{1}H-PANIC correction of NOE intensities in ^{1}H-observed 2D ^{1}H-^{19}F HOESY experiments, demonstrated here on several different fluorinated drug-like molecules of varying complexity and flexibility. We show the importance of accounting for the cross relaxation of ^{1}H in order to determine accurate values for ^{1}H-^{19}F internuclear distances and that the diagonal peaks of the 2D ^{1}H-^{1}H NOESY can be used to correct for this ^{1}H cross relaxation. In these systems correcting for ^{19}F relaxation is less helpful, presumably due to the more homogeneous T_1 times of these nuclei, at least in the examples examined here. The benefit of this increased accuracy is demonstrated in stereochemical elucidations of two of the test cases, where PANIC-correction offers a clear and superior discrimination between the rival structures.

Acknowledgments

The authors would like to thank Genentech and the Bristol Chemical Synthesis Doctoral Training Center for financial support.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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